Regular Article

Acyl-glucuronide as a Possible Cause of Trovafloxacin-Induced Liver Toxicity: Induction of Chemokine (C-X-C Motif) Ligand 2 by Trovafloxacin Acyl-glucuronide

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Trovafloxacin is an antibiotic that was withdrawn from the market relatively soon after its release due to the risk of hepatotoxicity. Trovafloxacin is mainly metabolized to its acyl-glucuronide by uridine 5'-diphosphate (UDP)-glucuronosyltransferase (UGT) 1A1. In this study, we examined whether the acyl-glucuronide is involved in the development of hepatotoxicity. A UGT1A1-induced cell model was developed and the toxicity of trovafloxacin acyl-glucuronide was evaluated. The UGT1A1-induced cell model was developed by treating HepG2 cells with chrysine for 48 h. Chemokine (C-X-C motif) ligand 2, a cytokine involved in drug-induced liver injury, was uniquely induced in drug-induced liver injury, was uniquely induced by trovafloxacin in the UGT1A1-induced HepG2 cells. Induction of UGT1A1 resulted in a decrease in cell viability. An in vivo animal study further demonstrated the importance of the UGT1A1 in the trovafloxacin-induced liver toxicity. Although the complete mechanism of trovafloxacin-induced liver injury is still unknown, trovafloxacin acyl-glucuronide can be involved in the development of toxic reactions in vitro and in vivo.

Key words trovafloxacin; drug-induced liver injury; acyl-glucuronide; chrysine; HepG2 cell

Uridine 5'-diphosphate (UDP)–glucuronosyltransferases (UGTs; EC 2.4.1.17) are a family of membrane-bound enzymes that catalyze glucuronidation of endogenous and exogenous compounds by transferring the glucuronic acid moiety of UDP-glucuronic acid to the substrates. Human UGTs are mainly divided into two distinct families, UGT1 and UGT2, on the basis of evolutionary divergence and homology. The UGT1 gene is located on chromosome 2q37 and produces nine functional enzymes, UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10, by exon sharing. The unique first exons encode N-terminal domain and the common exons 2 to 5 encode C-terminal domain of UGT1A proteins. Since all of UGTs recognize and utilize UDP-glucuronic acid as a co-substrate, it has been suggested that the C-terminal domain is responsible for the co-substrate binding. In contrast, UGT1A proteins exhibit overlapping but distinct substrate specificities, suggesting that the N-terminal domain is responsible for the substrate binding. While the liver is the most contributing tissue to the metabolism, recent findings suggest that extrahepatic tissues such as small intestine play an important role in glucuronidation of endogenous and exogenous compounds.

Trovafloxacin is an antibiotic that was released on the market in 1998. This promising agent was withdrawn from the market relatively soon after its release due to the risk of hepatotoxicity including acute liver failure. Trovafloxacin is mainly metabolized by UGTs to its acyl-glucuronide in humans. While glucuronides are usually pharmacologically inactive, certain types of glucuronide, especially acyl-glucuronide, can exhibit an increased reactivity compared to the parent compounds. Acyl-glucuronide-associated toxicity has been reported in vivo and in vitro; however, there are also reports showing that acyl-glucuronidation did not induce the cyto- and genotoxicity. Microarray expression analysis is a promising tool to identify genes associated with a drug treatment. To identify genes that were associated with the trovafloxacin-induced liver toxicity, several research groups carried out the microarray expression analysis in human hepatocytes, mice, and rats. The group of genes that were specifically induced by the trovafloxacin treatment included topoisomerase I (TOPI), B-cell leukemia/lymphoma 2 (BCL-2)-associated transcription factor 1 (BCLAF), Mitofusin1 (MFN1), Metallothionein (MT) 2A, MT1H, and MT1X. Although these genes might have been induced by the acyl-glucuronide in the hepatocytes, there still was a possibility that the parent compound itself was involved in the induction of the genes. This was because a certain amount of trovafloxacin still remained in the body even 24 h after the oral and intravenous administration of trovafloxacin.

Previously, we determined UGT1A1 as the main UGT isoform responsible for trovafloxacin acyl-glucuronidation. To investigate whether trovafloxacin acyl-glucuronide is involved in trovafloxacin-induced liver injury, in the present study, a UGT1A1-induced cell model was developed and the toxicity of trovafloxacin acyl-glucuronide was evaluated. We further employed Ugt1-knockout mice to examine the importance of trovafloxacin acyl-glucuronide in the trovafloxacin-induced liver injury in vivo.

MATERIALS AND METHODS

Chemicals and Reagents UDP-glucuronic acid (UDPGA), alamethicin, chrysine, estradiol, and estradiol 3-O-glucuronide were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Trovafloxacin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Recombinant human

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tumor necrosis factor α (TNF-α) was purchased from Roche (Mannheim, Germany). Primers were commercially synthesized at Life Technologies (Carlsbad, CA, U.S.A.). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

**Cell Culture and Chemical Treatments** The human hepatoma HepG2 cells were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). HepG2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS) and were maintained at 37°C in a humidified atmosphere containing 5% of CO₂. Before the treatment, HepG2 cells were seeded into six-well plates at 5×10⁵ cells/well. After 24 h. the culture medium was changed to a normal or a chrysin-containing DMEM medium and subsequently cells were maintained for 48 h until harvesting. RNA was isolated from the cells and was used for the quantitative-reverse-transcription PCR (Q-PCR) analysis. The microsomal fraction was also obtained from the cells. Control and the UGT1A1-induced HepG2 cells were further treated with trovafloxacin (50 µM) for 24 h.

**Q-PCR Analysis** cDNA was synthesized from total RNA using ReverTra Ace qPCR RT Master Mix (Toyobo, Tokyo, Japan) according to the manufacturer’s protocol. Q-PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo), and the reactions were run in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, U.S.A.). Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control for the cDNA quantity and quality. Primer pairs that can detect UGT1A1 and GAPDH were reported previously.

Results of the UGT1A1-Induced HepG2 cells were analyzed by the manufacturer’s protocol for 24 h. Forty-eight hours after the treatment, RNA was isolated and a Q-PCR analysis was carried out to determine the UGT1A1 expression level in the cells. We observed that phenytoin, carbamazepine, and phenobarbital moderately induced UGT1A1 expression in HepG2 cells. In contrast, it was demonstrated that chrysin significantly induced UGT1A1. In a subsequently performed study, chrysin concentration-dependently induced the UGT1A1 mRNA expression in HepG2 cells (Fig. 1A). The highest expression of UGT1A1 was observed when the cells were treated with 50-µM chrysin. When HepG2 cells were treated with even higher concentrations of chrysin such as 80 and 100 µM, slight and moderate cytotoxicity was induced, respectively. It was further demonstrated that the microsomes prepared from the HepG2 cells treated with 50-µM chrysin exhibited an 8-fold higher estradiol 3-O-glucuronidation activity (Fig. 1B). The estradiol 3-O-glucuronidations in the cells were also determined in the absence of additional UDPGA. Estradiol was added into the cell-culturing media and was incubated for 7 h. Estradiol 3-O-glucuronide was detected in the cell-culturing medium of HepG2 cells. The amount of glucuronide was three-fold greater in the medium of the UGT1A1-
induced HepG2 cells (Fig. 1C). Trovafloxacin acyl-glucuronide was not detected in the reaction mixture including the microsomes prepared from the control HepG2 cells. Meanwhile, a slight but detectable amount of trovafloxacin acyl-glucuronide was observed in the reaction mixture including the microsomes prepared from the UGT1A1-induced HepG2 cells with 1.1 pmol/min/mg (Fig. 1D). Estradiol is a selective substrate of UGT1A1; therefore, it was demonstrated that the HepG2 cells treated with 50-µM chrysin was the UGT1A1-induced cell model.

Expression of Trovafloxacin-Induced Genes in HepG2 Cells

It has been shown that TOP1, BCLAF, MFN1, MT2A, MT1H, and MT1X were significantly induced in the liver when hepatocytes and animals were treated with trovafloxacin. To investigate whether these trovafloxacin-associated genes are induced by trovafloxacin acyl-glucuronide or not, the mRNA expression of the genes was quantified in the HepG2 cells and the cells treated with trovafloxacin in the presence or absence of chrysin. It was confirmed that chrysin highly induced UGT1A1 in the HepG2 cells, while trovafloxacin itself did not induce UGT1A1 (Fig. 2A). MT2A, TOP1, and BCLAF were induced 2- to 3-fold by trovafloxacin (Fig. 2B), which was in agreement with the previous reports. In the UGT1A1-induced cells, however, the expression level of MT2A, TOP1, and BCLAF was still 2- to 3-fold higher in the presence of trovafloxacin compared to the level in the control cells. Increased amount of UGT1A1 in the HepG2 cells did not affect the expression of MT2A, TOP1, and BCLAF mRNA, suggesting that trovafloxacin acyl-glucuronide was not involved in the induction of these genes. The expression levels of MFN1, MT1H, and MT1X were also examined in the control and UGT1A1-induced HepG2 cells; however, none of these genes were specifically induced by trovafloxacin in the UGT1A1-induced HepG2 cells (Fig. 2B).

Expression of Toxicity-Associated Genes in HepG2 Cells

It has been demonstrated that neuronal apoptosis inhibitory protein, MHC class II, HET-E, telomerase protein 1 domain-, leucine-rich repeat-, and pyrin domain-containing protein 3 (NALP3), receptor for advanced glycation endproducts (RAGE), interleukin (IL)-6, and IL-23p19 were highly induced by drugs that were associated with drug-induced liver injury (DILI) in the cell-based assay. In HepG2 cells, IL-6 was induced 2-fold when HepG2 cells were treated with trovafloxacin (Fig. 2C). Meanwhile, trovafloxacin did not induce NALP3, RAGE, or IL-23p19 in HepG2 cells. In the UGT1A1-induced model, such induction pattern was still the same as observed in the control HepG2 cells.

Chemokine (C-X-C motif) ligand 2 (CXCL-2), S100A9, and IL-1β are also genes associated with DILI. To further determine whether these genes are specifically responsive to trovafloxacin acyl-glucuronide, the expression levels of CXCL-2, S100A9, and IL-1β were examined in the control and
UGT1A1-induced HepG2 cells. CXCL-2 was induced 4-fold by the treatment of trovafloxacin in the HepG2 cells (Fig. 2D). In the UGT1A1-induced cells, trovafloxacin induced CXCL-2 more than 10-fold compared to the level in the control cells. S100A9 was not induced by trovafloxacin; however, this gene was highly induced by chrysin (Fig. 2D). IL-1β was similarly
induced by chrysin in HepG2 cells in the presence or absence of trovafloxacin (Fig. 2D). CXCL-2 was uniquely induced by trovafloxacin in the UGT1A1-induced HepG2 cells, indicating that CXCL-2 might be specifically induced by trovafloxacin acyl-glucuronide.

Importance of UGT1A1 in the Trovafloxacin-Induced Liver Toxicity in Vitro and in Vivo The effect of the UGT1A1 induction on cell viability was examined by an MTT assay. Co-treatment of control HepG2 cells with trovafloxacin and TNF-α decreased the cell viability by 50% (Fig. 3A, left column). When UGT1A1-induced HepG2 cells were used, the co-treatment decreased the cell viability by 65% (Fig. 3A, right column). To further investigate the importance of UGT1A1 in the trovafloxacin-induced liver toxicity in vivo, we utilized Ugt1−/- knockout mice. It was previously shown that Ugt1 knockout mice display no UGT1 activities, while wild type and heterozygous Ugt1 mice similarly have higher glucuronidation activities. When wild type and heterozygous Ugt1 mice were co-treated with trovafloxacin as well as LPS, serum ALT levels were increased 5-fold. In contrast, such increase was not observed in the Ugt1 knockout mice (Fig. 3B). Although it was not statistically significant, these data indicate that UGT1A1 might be involved in the trovafloxacin-induced hepatotoxicity in vitro and in vivo.

DISCUSSION

DILI is one of the leading causes of acute liver failure in the U.S.A. While trovafloxacin was withdrawn from the market due to the high risk of developing the severe liver damage, the molecular mechanism underlying the trovafloxacin-induced hepatotoxicity remains to be cleared. Importantly, whether the reactive metabolite—trovafloxacin acyl-glucuronide—is involved in the toxic reaction or not has been inconclusive. Various cell-based assays have been developed to evaluate the cyto- and genotoxicity of compounds. An addition of liver microsomes or drug-metabolizing enzyme-expressing systems, as well as the substrates, into the cell-culturing medium is a convenient method to generate reactive metabolites in the cell-culturing medium. In fact, this method can be used to determine the hepatotoxic potential of compounds in preclinical drug development. Since most of drug-metabolizing enzymes are localized in the endoplasmic reticulum membrane, extremely hydrophilic metabolites such as glucuronides are usually produced inside the cells. Meanwhile, such metabolites would be generated outside of the plasma membrane if liver microsomes and enzyme-expressing systems were experimentally added into the medium. To properly evaluate the effect of trovafloxacin acyl-glucuronide on the hepatic cells, in the present study, we developed UGT1A1-induced HepG2 cells.

Due to the detection limit of the instruments used, we were not able to determine the formation of trovafloxacin acyl-glucuronide in the cells. However, the microsomes prepared from the UGT1A1-induced HepG2 cells exhibited higher estradiol 3-O- and trovafloxacin acyl-glucuronidation activities (Figs. 1B, D), indicating that a higher amount of trovafloxacin acyl-glucuronide should have been produced in the UGT1A1-induced cells. A previous study reported that trovafloxacin was mainly metabolized to its glucuronide in humans, while it is partially metabolized to N-acetyltrovafloxacin and sulfate conjugate. Therefore, it was considered that the effect of other trovafloxacin-metabolizing enzymes and their metabolites on the trovafloxacin-induced cytotoxicity was minor. Thus, it is assumed that the 10-fold induction of CXCL-2 (Fig. 2D) was specifically caused by trovafloxacin acyl-glucuronide.

In vitro MTT assays and in vivo studies with Ugt1 knockout mice also indicated that UGT1A1 was highly involved in the trovafloxacin-induced hepatotoxicity (Fig. 3). C-X-C Motif chemokine receptor 2 (CXCR2) is a receptor of CXCL-2. It was previously demonstrated that ischemia–reperfusion caused significant liver injury in wild type mice, but not in CXCR2-deficient mice. Moreover, it was demonstrated that a treatment of primary hepatocytes with recombinant CXCL-2 induced lactate dehydrogenase (LDH) release in the cells. Induction of CXCL-2 was also observed in mice treated with hepatotoxic a-naphthylisothiocyanate, carbon tetrachloride, and acetaminophen. Previously, it was indicated that certain types of cells and cytokines, such as Th17 cells and TNF-α, are commonly involved in various drug-induced hepatotoxicity. Therefore, CXCL-2 can also be specifically induced by certain toxicants and its induction might play a significant role in the development of liver injury. The detailed mechanism underlying the induction of CXCL-2 by trovafloxacin acyl-glucuronide needs to be elucidated in the future. Since Toll like receptor 2 (TLR2) is tightly associated with the develop-
ment of liver injury as well as the gene expression of hepatic CXCL-2, TLR2 might be the key factor in trovafloxacin acyl-glucuronide-associated liver injury.

In conclusion, we treated HepG2 cells with chrysin, a known UGT inducer, to induce UGT1A1. CXCL-2, a cytokine involved in DILI, was uniquely induced by trovafloxacin in the UGT1A1-induced HepG2 cells. In vitro and in vivo studies further demonstrated the importance of UGT1A1 in the trovafloxacin-induced liver toxicity. Although the complete mechanism of trovafloxacin-induced liver injury is still unknown, trovafloxacin acyl-glucuronide can be involved in the development of toxic reactions in vitro and in vivo. Interindividual variability in the UGT1A1 activity as well as protein–protein interactions involving UGT might be associated with the idiosyncrasy of trovafloxacin-induced liver injury in humans.

Conflict of Interest  The authors declare no conflict of interest.

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